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(57) Abstract

The present invention discloses sphingoid base derivatives which are salts of a sphingoid base. These salts have a substantially increased solubility in an aqueous environment and display an increased efficacy in compositions for topical use.

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SPHINGOID BASE DERIVATIVES AND USES THEREOF

Field of the invention

The present invention relates to the field of topical application, especially topical application of sphingoid base derivatives.

Background of the invention

Sphingoid bases like sphingosine are known to be potent effectors of skin cell differentiation and proliferation, by interfering with basic biochemical cell processes (Hannun, Y.A. and Bell, R.M. (1989), Science 243, 500-507). For example, free sphingosine inhibits the activity of protein kinase C and thus plays a pivotal role in signal transduction and regulation of cell division (Hannun, Y.A. et al. (1986), J. Biol. Chem. 261, 12604-12609). The action of free sphingosine may be an important factor in the modulation of epidermal cell proliferation in order to balance the rate in which cells are lost from the skin surface (Downing, D.T. (1992) J. Lipid Res., 33, 301-313). In addition, other biological activities have been described for sphingoid bases, such as antimicrobial activity (Bibel, D.E. et al. (1992), J. Invest. Dermatol. 98, 269-273).

Due to their effect on skin cell differentiation and proliferation and their antimicrobial activity, sphingoid bases may be included as an active ingredient in various cosmetic compositions. For example, sphingosine has been described for the treatment of various abnormalities and disorders concerning the skin, such as dry skin, xeroderma and psoriasis. Sphingosine can also protect the skin against various harmful or undesirable effects, such as the effects of UV light and skin ageing. In particular, sphingoid bases have been included in topical compositions as an antiinflammatory agent or an antimicrobial agent (WO98/49999).

A disadvantage of sphingoid bases is their scarce solubility in an aqueous environment. This phenomenon hampers the use of these compounds in aqueous formulations. For instance, to display an effective antimicrobial activity, it is important to have the sphingoid base solubilized in an aqueous formulation.

Description of the invention

The present invention discloses derivatives of sphingoid bases which have a substantially increased solubility in water than their free base counterparts. As a consequence, these sphingoid base derivatives display a surprisingly improved efficacy when formulated in an aqueous composition.

The sphingoid base derivatives of the invention are salts of sphingoid bases.

According to the invention, the anion of a sphingoid base salt is derived from any suitable acid. In that regard, a suitable acid is defined as an acid which, upon mixing with a sphingoid base in a suitable solvent, produces a salt which has an increased solubility in an aqueous medium as compared to the solubility of the sphingoid base as such.

In one embodiment of the invention, the acid is an acid which itself may have an efficacy in topical application.

In one embodiment of the invention, the acid is a hydrophilic acid able to deliver the sphingoid base to the water phase of a cosmetic or pharmaceutical composition.

Preferably, the acid is a hydrophilic organic acid such as an α-hydroxy alkanoic acid, a β-hydroxy alkanoic acid, an α,β-dihydroxy alkanoic acid, an alkanedioic acid or a mineral acid. More preferred examples of hydrophilic organic acids are lactic acid, glycolic acid, malic acid, pyruvic acid, succinic acid, fumaric acid, citric acid, ascorbic acid, gluconic acid and/or pyroglutamic acid (pyrrolidone carboxylic acid). More preferred examples of mineral acids are hydrochloric acid, nitric acid and/or phosphoric acid.

In another embodiment of the invention, the acid is a lipophilic organic acid, such that the combination with a sphingoid base increases the efficacy of both the lipophilic acid as well as the sphingoid base.

The sphingoid base salts of the invention may be prepared as follows. The sphingoid base is dissolved in a suitable organic solvent, whereupon at least one equivalent of a suitable acid is added. Typically, addition of the acid will result in a decrease in the pH of at least about 3 units. It is appreciated that the final value of the pH will be dependent on the acid being applied. Dissolution of the sphingoid base in the organic solvent preferably occurs at an elevated temperature, for instance a temperature of 50°C to 70°C. Upon cooling of the

mixture, the sphingoid base salt will precipitate. The crystalline precipitate is recovered from the reaction mixture by filtration and optionally may be washed with solvent, preferably the same solvent as being used for preparation of the salt.

A suitable organic solvent preferably is a solvent wherein the end product, i.e. the sphingoid base salt, is insoluble. A suitable organic solvent for instance is ethanol or methyl isobutyl ketone.

In one embodiment of the invention, a sphingoid base salt is used as a starting compound for the preparation of another sphingoid base salt.

It is essential that the sphingoid base salts of the invention are prepared prior to their intended use, e.g. their inclusion in a topical composition. The inclusion of a free sphingoid base in a topical composition additionally containing anions from one or more of the acids as defined herein above will not result in an increased solubility and/or an increased efficacy.

The sphingoid base salts of the invention preferably are salts of the sphingoid bases sphingosine, sphinganine or phytosphingosine. More preferably, the sphingoid base salts are salts of phytosphingosine.

In one embodiment of the invention, phytosphingosine is obtained via a microbial fermentation. For instance, phytosphingosine is obtained from Pichia ciferrii-derived tetraacetyl-phytosphingosine (TAPS), by a suitable deacetylation reaction. The deacetylation may be chemical, e.g. by base catalyzed hydrolysis with potassium hydroxide, or enzymatical. After alkaline hydrolysis of TAPS, the resulting phytosphingosine may be purified. Such a purification can occur by any method known to a person skilled in the art. Yeast-derived phytosphingosine is human skin-identical, as it is reported to have the same stereochemical configuration as mammalian phytosphingosine, i.e. the D-D-erythro configuration.

The sphingoid base salts of the invention have a solubility in an aqueous environment which is considerably higher than the solubility of the free sphingoid base. It is further surprisingly shown by the present invention that sphingoid base salts have an increased efficacy as compared to the free sphingoid base, even in an environment where the free sphingoid base also is in a solubilised form. The free sphingoid base may be in a solubilised form by the additional presence in the aqueous medium of an organic solvent and a surface active compound.

Compositions comprising a sphingoid base derivative according to the invention are suitable for topical application, whereby topical application is understood to comprise cosmetic and/or dermatological application on the skin, on hair and on the epithelial linings of mouth, nose, eye, urogenital tract, and the like.

The sphingoid base derivatives of the present invention preferably are incorporated in a topical composition in a concentration which may range from 0.001 to 5 wt %, preferably from 0.005 to 5 wt %, more preferably from 0.01 to 2.5 wt %, most preferably from 0.02 to 1 wt %, especially preferably from 0.02 to 0.5 wt %.

Topical compositions including a sphingoid base derivative according to the invention are particularly suitable to apply to various topically occurring undesirable and/or abnormal conditions associated with inflammation and/or microbial activity.

Examples of topically occurring undesirable and/or abnormal conditions to which topical compositions comprising the sphingoid base derivatives of the invention are advantageously applied are eczema, psoriasis, atopic dermatitis, acne, dandruff, mouth and/or lip infections, mycoses, various other skin-infectious diseases or vaginal infections. Topical compositions comprising said sphingoid base derivatives are further advantageously applied for wound-healing, e.g. in case of burns, and for normalisation of skin flora.

Due to their antimicrobial activity, the sphingoid base derivatives of the invention additionally may function as a preservative in cosmetic and dermatological compositions, to decrease and/or substitute for existing chemical preservatives.

Example 1

Preparation of PS.lactate

A mixture of 50 grams of phytosphingosine and 500 ml of absolute ethanol was stirred and heated to 65°C. Next the almost clear solution was filtered while hot through a paper filter into a 1 litre 3-necked flask.

While stirring (L)-lactic acid (25.7 g) was added to the filtrate in portions to decrease the pH from 9.9 to 5.3, while the temperature went up from 66°C

to 71°C. The mixture was stirred and cooled. At ca 45°C crystallisation started, while cooling was continued over a period of 3/4 hour to 21°C.

The precipitate was filtered off and the cake was replaced with 150 ml of ethanol (fast filtration and replacing, total of 2 minutes).

The wet cake (110.8 g) was dried under vacuum to give 51.2 grams of product. NMR analysis gave a purity of 99.3%.

Example 2

Preparation of PS.glycolate

A mixture of 50 grams of phytosphingosine and 500 ml of absolute ethanol was stirred and heated to 65°C. Next the almost clear solution was filtered while hot through a paper filter into a 1 litre 3-necked flask. Rinsed with 20 ml of hot ethanol. The filtrate was heated again to 65°C.

While stirring glycolic acid (13.4 g) was added to the filtrate in portions to decrease the pH from 9.9 to 5.6, while the temperature went up from 65°C to 68°C. The mixture was stirred and cooled. At ca 66°C crystallisation started, while cooling was continued over a period of 20 minutes to 25°C.

The precipitate was filtered off and the cake was replaced with 150 ml of ethanol (fast filtration and replacing, total of 3 minutes)

The wet cake (87 g) was dried overnight under vacuum to give 56.6 grams of product. NMR analysis gave a purity of 98.6%.

Example 3

Preparation of PS.HCl

A mixture of 50 grams of phytosphingosine and 500 ml of absolute ethanol was stirred and heated to 65°C. Next the almost clear solution was filtered while hot through a paper filter into a 1 litre 3-necked flask. Rinsed with 20 ml of hot ethanol.

While stirring hydrochloric acid (36%, ca 13 ml) was added to the filtrate to decrease the pH from 10.3 to ca 0, while the temperature went up from 45°C to 50°C. The mixture was stirred and cooled. At 34°C crystallisation started after seeding and cooling was continued over a period of 0.5 hour to10°C.

The precipitate was filtered off and the cake was replaced with 100 ml of cold ethanol (slow filtration and replacing, total of 3/4 hour.)

The wet cake (272g) was dried under vacuum to give 48.0 grams of product. NMR analysis gave a purity of 96.7%.

Example 4

Preparation of PS.pyroglutamate

A suspension of 25 grams of phytosphingosine, 200 ml of methyl isobutyl ketone (MIK) and 2 ml of water was stirred and heated to 66 °C.

Next 12 grams of DL-pyroglutamic acid were added, changing the pH from 9.4 to 5.8

A glassy precipitate was obtained. At 45 °C a 1 ml sample was taken which started to crystallise upon scratching. This was used to seed the mixture during further cooling.

Next the mixture was further cooled to 17 °C and filtered over a glass G3 filter, rinsed / replaced with 50 ml of fresh MIK (fast filtration). The wet cake (57 g) was dried in vacuum to give 34.3 grams of product.

Example 5

Preparation of PS.citrate

A suspension of 25 grams of phytosphingosine, 200 ml of methyl isobutyl ketone (MIK) and 1 ml of water was stirred and heated to 72 °C.

Next 18 grams of citric acid monohydrate were added, changing the pH from 9.4 to 1.8

A precipitate was obtained. Next the mixture was cooled to 14 °C and filtered over a glass G3 filter, rinsed / replaced with 50 ml of fresh MIK (fast filtration). The wet cake (84 g) was dried in vacuum to give 39.7 grams of product. NMR analysis gave a purity of 96.4 %.

Example 6

Antimicrobial activity of PS against yeasts

Two different yeast strains were used: *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10231. All incubations were either performed at 30°C (for S. cerevisiae) or at 37°C (for C. albicans). Both yeast strains were grown in YEPD2% medium (20 g/l glucose, 10 g/l peptone, 20 g/l yeast extract, pH = 6.0). The cultures were grown overnight, the cells in 50 µl of culture were harvested by centrifugation, washed with 1 ml sterile buffer (10 mM HEPES (pH = 7.2 with NaOH) + 20 g/l glucose), centrifuged, and resuspended in 0.5 ml sterile buffer.

A 10 mg/ml stock solution of phytosphingosine (PS) was prepared in a solvent system, consisting of 1 volume fraction ethanol, 2 volume fractions Tween 20 and 17 volume fractions 50% glycerol in water. The components of the solvent system were added to the phytosphingosine in this order, and the solution was shaken vigorously after each addition. When all solvents had been added, the mixture was heated to 40°C for 15 to 30 minutes. Dilutions from this stock solution (if necessary) were made in 5% ethanol in water. All solutions were prepared 24 hours prior to use, and kept at room temperature.

The antifungal effect of phytosphingosine against these two yeasts was investigated using the LIVE/DEAD® Yeast Viability Kit L-7009 (Molecular Probes Inc., Oregon, USA). This kit employs two different fluorescent stains, FUN-1™ and Calcofluor™ White M2R, to make distinction between living and dead cells, which can be observed using a fluorescence microscope with the appropriate filters.

For this analysis, the following amounts of fluorescent dyes were added to 0.5 ml yeast cell suspension in sterile buffer, prepared as described previously: 1 µl FUN-1™ and 2.5 µl Calcofluor™ White M2R. After mixing, these suspensions were incubated for 30 minutes. Then 50 µl of an appropriate dilution of the phytosphingosine stock solution were added, to obtain the final concentrations indicated in Figures 1a and 1b. After mixing, these suspensions were incubated, and the fraction of living and dead cells was followed over time.

An Olympus BHB fluorescence microscope was used for the microscopic observations, with two different filter sets:

1. Dichroic mirror blue (B), excitation filter IF490, emission filter O530 (living cells show orange particles in the cell, whereas dead cells are evenly coloured green/orange).
2. Dichroic mirror violet (V), excitation filters U95-B93, emission filter Y455 (living cells show blue cell walls, whereas dead cells do not).

The results are shown in Figure 1a and 1b. It is clear that both yeast strains were killed by phytophingosine (PS) in a dose-dependent fashion.

Example 7

Antifungal action of PS towards starved cells

In their natural habitat, microbial cells are in a starved condition for most of the time. This prompted us to investigate whether the antimicrobial action of PS would also be manifest against starving cells.

To this end, the procedure described in Example 6 was slightly modified (all methods and conditions were identical unless specified otherwise). Cells from an overnight culture of *Candida albicans* ATCC 10231 were harvested by centrifugation. Two different buffers were used to wash and resuspend the cells: 10 mM HEPES (pH= 7.2 with NaOH) + 20 g/l glucose as used in Example 6, and the same buffer without glucose.

The two cell suspensions, with and without glucose (2.5 ml for each condition), were incubated for 10 minutes at 37°C. Then 125 µl of an appropriate dilution of the phytosphingosine stock solution were added, to obtain the final concentrations indicated in Figure 2. After mixing, these suspensions were incubated further, and at the indicated time points 100 µl samples were drawn. The cells were harvested by centrifugation, and the cells were resuspended in 100 µl buffer with glucose, and with the same concentrations of fluorescent dyes as in Example 6. This analysis mixture was incubated for 10 minutes more, to allow the dyes to be taken up into the cells, and the numbers of living and dead cells were determined as described in Example 6.

As is shown in Figure 2, the starved cells were more susceptible to the antifungal action of PS than were the energised cells. In fact, after an initial lag

phase, 250 mg/l PS proved to be as effective in killing the starved cells as 500 mg/l. The lag phase is presumably due to the presence of endogenous energy stores of the cells, and this again shows that PS is particularly effective towards the starved condition.

Example 8

Antibacterial effect of PS in cosmetic preparations in an agar diffusion test

An overnight culture of Staphylococcus aureus ATCC 14458 was prepared similarly to the procedure described in Example 6 (BHI medium (Difco), incubation at 37°C).

To prepare agar plates for the diffusion test, 300 ml of BHI medium, with 1% agar and 15% glycerol added, was molten and cooled to 50°C. Then 6 ml of a sterile glucose solution (50% w/v) and 6 ml of the overnight culture of the micro-organism were added. Petri dishes were filled with 12.5 ml of this agar medium, and the medium was allowed to solidify.

The test formulations were prepared as indicated in Figure 6, with a lipid phase of octyl dodecyl lactate. They contained 1, 2 or 5 g/l PS.

To apply a test sample to the test plates, a stainless steel ring (6 mm internal diameter) was put into an empty, sterile Petri dish. Inside this ring, 2 paper disks (6 mm diameter) were placed to cover the bottom, and 50 µl of the test formulation was applied to the filter disks. The rings were put on the surface of the agar plates containing the micro-organisms, the agar plates were stored at 5°C for the periods indicated in the Table to allow diffusion of the test solutions, after which the rings were removed. Subsequently, the agar plates were incubated at a temperature suitable for the growth of the micro-organisms (37°C). After the micro-organisms were fully grown in the non-inhibited areas of the agar plates, the degree of inhibition was measured as the zone of no growth (or diminished growth) extending from the area of application in two orthogonal directions.

Table 1. Antibacterial effect of PS in a cosmetic preparation

[PS] (g/l)	Diffusion 5 days 5°C			Diffusion 14 days 5°C		
1	-	-	-	-	-	-
2	7	6	5	7/14	7/13	5/11
5	5	6.5	7	7.5/14	9.5/16	9/15

Where two figures are given, the first refers to the zone of no-growth, whereas the second refers to the zone of diminished growth.

As appears from Table 1, there is a dose-dependent growth inhibition by PS.

Example 9

Antifungal action of PS and some of its derivatives

It was found that some derivatives of phytosphingosine had an improved solubility in aqueous systems. This prompted us to compare their antimicrobial activity to that of PS itself. The following derivatives were investigated: the glycolic acid, lactic acid and hydrochloric acid salts of phytosphingosine. Stock solutions of the phytosphingosine salts were prepared as described in Example 6 for phytosphingosine, and the same experimental conditions were used.

It was found that all three salts tested had a much stronger antifungal activity than had the free base. This was not due to the anions present in the salt solutions, since blanks with the appropriate amounts of lactate, chloride or glycolate were without effect (Figure 3a). In Figure 3b it can be seen that the potency of the chloride was more than 2.5 times higher than that of the PS base.

Example 10

Antifungal action of PS and some of its derivatives in a solvent-free system

Solutions in demineralised water were prepared similarly to the procedure described for the solvent system (Example 6), including the final heating step.

In Figure 4a it can be seen that the lack of solvents during sample preparation all but abolished the antifungal effect of the free PS base, whereas the potency of the PS salts was not decreased. In fact, it was found that the potency of the PS salts can even be higher in the solvent-free system, as is shown in Figures 4b and 4c.

Example 11

Antibacterial effect of PS against bacteria

Two different bacterial strains were used: *Staphylococcus aureus* ATCC 14458 and *Escherichia coli* 421. All incubations were performed at 37°C. Both bacteria were grown as described in Example 8, 50 µl culture was harvested by centrifugation, washed with 1 ml sterile demineralised water, and resuspended in 0.5 ml sterile demineralised water.

A 10 mg/ml stock solution of PS was prepared as described previously. Dilutions from this stock solution (if necessary) were made in 5% ethanol in water. All solutions were prepared 24 hours prior to use, and kept at room temperature.

The antibacterial effect of phytosphingosine against these two bacteria was investigated using the LIVE/DEAD® BacLight™ Bacterial Viability Kit L-7012 (Molecular Probes Inc., Oregon, USA). This kit employs two different fluorescent stains, SYTO 9 stain and propidium iodide, to make distinction between living and dead cells, which can be observed using a fluorescence microscope with the appropriate filters.

The solutions of the dyes provided in the kit were mixed 1:1 just before use, and 1.5 µl of this fluorescent dye mixture was added to 0.5 ml bacterial suspension. Subsequently, 50 µl of an appropriate dilution of the phytosphingosine stock solution were added, to obtain the final concentrations indicated in Figure 5. After mixing, these suspensions were incubated, and the fraction of living and dead cells were followed over time.

The microscopic examinations were performed using an Olympus BHB fluorescence microscope, with the following filter set: dichroic mirror blue (B),

excitation filter IF490, emission filter O530 (living cells are green, dead cells are orange/yellow).

It was found that *S. aureus* was strongly killed. However, this effect could not be quantified, since the dead cells were not detectable, due to lysis: the measurement with fluorescent dyes requires that the dead cell remains structurally intact. Therefore, the killing effect was apparent only in a strong decrease in the number of living cells.

The *E. coli* cells were also killed quite effectively, and in this organism the dead cells could be quantified readily (Figure 5). It appears that the cells are killed by PS in a dose-dependent manner, and that the bacteria are more sensitive to this compound than are the fungi.

Example 12

Antibacterial and antifungal effect of PS derivatives in a diffusion test

An overnight culture of the indicated test organisms was prepared as described in Example 6 and 8, and the samples were applied to the test plates as described in Example 8. The samples were prepared as the appropriate dilutions from stock solutions, prepared as described previously. The agar plates were stored overnight at 5°C to allow diffusion on the test solutions, after which the sample rings were removed. Subsequently, the agar plates were incubated at a temperature suitable for the growth of the micro-organisms (37°C). After the micro-organisms were fully grown in the non-inhibited areas of the agar plates, the degree of inhibition was measured as the zone of no growth (or diminished growth) extending from the area of application in two orthogonal directions (in mm).

Table 2. Antibacterial and antifungal effects of PS-derivatives in an agar diffusion test

Derivative	(g/l)	S. aureus ATCC 14456			C. albicans ATCC10231		
PS-lactate	10	12	13	13	9*	9.5*	10.5*
	5	12	10.5	12	8*	9*	9*
	2	8	8.5	9	±	5*	5*
	1	6	5	5	4*	6*	4*
PS-glycolate	10	11.5	9.5	11	7.5	5*	6
	5	9	9	9	3	3	3
	2	7	6	5	-	-	-
	1	3	4.5	3	-	-	-
PS-chloride	10	12.5	11	11	7	6*	7
	5	9	9	9	2	3	3
	2	6	7	5	-	-	-
	1	5	5	5	-	-	-

* The data marked with an asterisk refer to a zone of inhibited growth; the unmarked data refer to zones of no growth.

It appears from Table 2 that the PS derivatives are fully active against bacteria and fungi in the diffusion test, and that there is a clear dose dependency of this effect.

CLAIMS

1. A sphingoid base derivative which is a salt of a sphingoid base.

2. The sphingoid base derivative of claim 1, wherein the anion of the salt is derived from a hydrophilic acid.

3. The sphingoid base derivative of claim 2, wherein the hydrophilic acid is a hydrophilic organic acid or a mineral acid.

4. The sphingoid base derivative of claim 3, wherein the hydrophilic acid is selected from the group consisting of lactic acid, glycolic acid, malic acid, pyruvic acid, succinic acid, fumaric acid, citric acid, ascorbic acid, gluconic acid and pyroglutamic acid.

5. The sphingoid base derivative of claim 3, wherein the hydrophilic acid is selected from the group consisting of lactic acid, glycolic acid, pyroglutamic acid, citric acid and hydrochloric acid.

6. A process for the preparation of the sphingoid base derivative of claim 1 comprising the addition of at least one equivalent of an acid to a solution of the sphingoid base in a suitable solvent and the recovery of the crystalline sphingoid base salt from the reaction mixture.

7. The process of claim 6, wherein the solvent is ethanol or methyl isobutyl ketone.

8. A composition for topical use comprising the sphingoid base derivative of claim 1.

9. The composition of claim 8 which is a cosmetic composition.

10. The composition of claim 8 or 9 comprising the sphingoid base derivative in a concentration ranging from from 0.001 to 5 wt %, preferably from 0.005 to 5 wt %, more preferably from 0.01 to 2.5 wt %, most preferably from 0.02 to 1 wt %, especially preferably from 0.02 to 0.5 wt %.

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11. A sphingoid base derivative according to claim 1 for use as a medicament.

12. Use of a sphingoid base derivative according to claim 1 for the manufacture of a medicament for use in antimicrobial and/or anti-inflammatory treatment.

Figure 1a. Antifungal effect of PS against S. cerevisiae

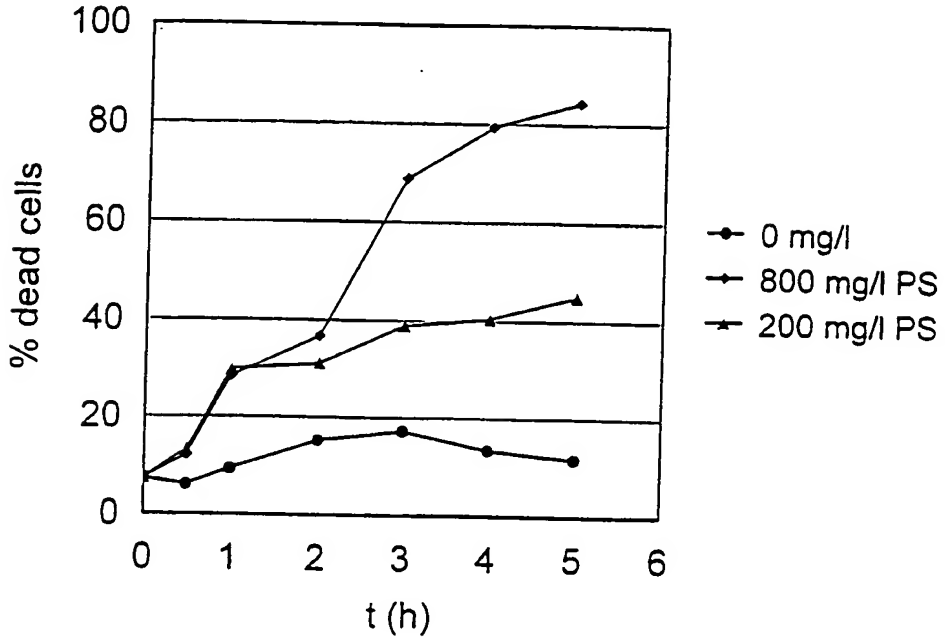
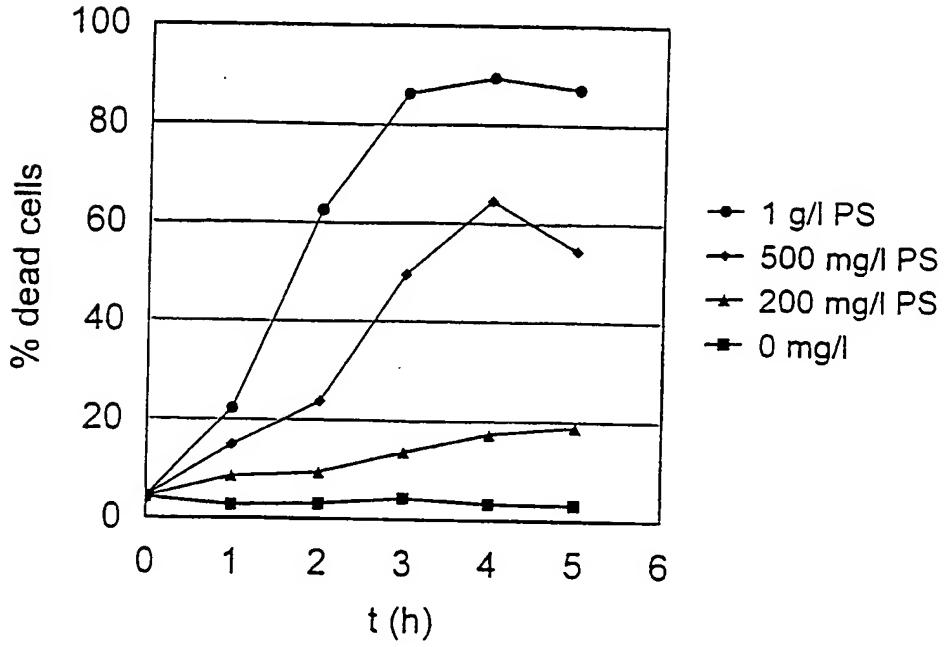


Figure 1b. Antifungal effect of PS against C. albicans



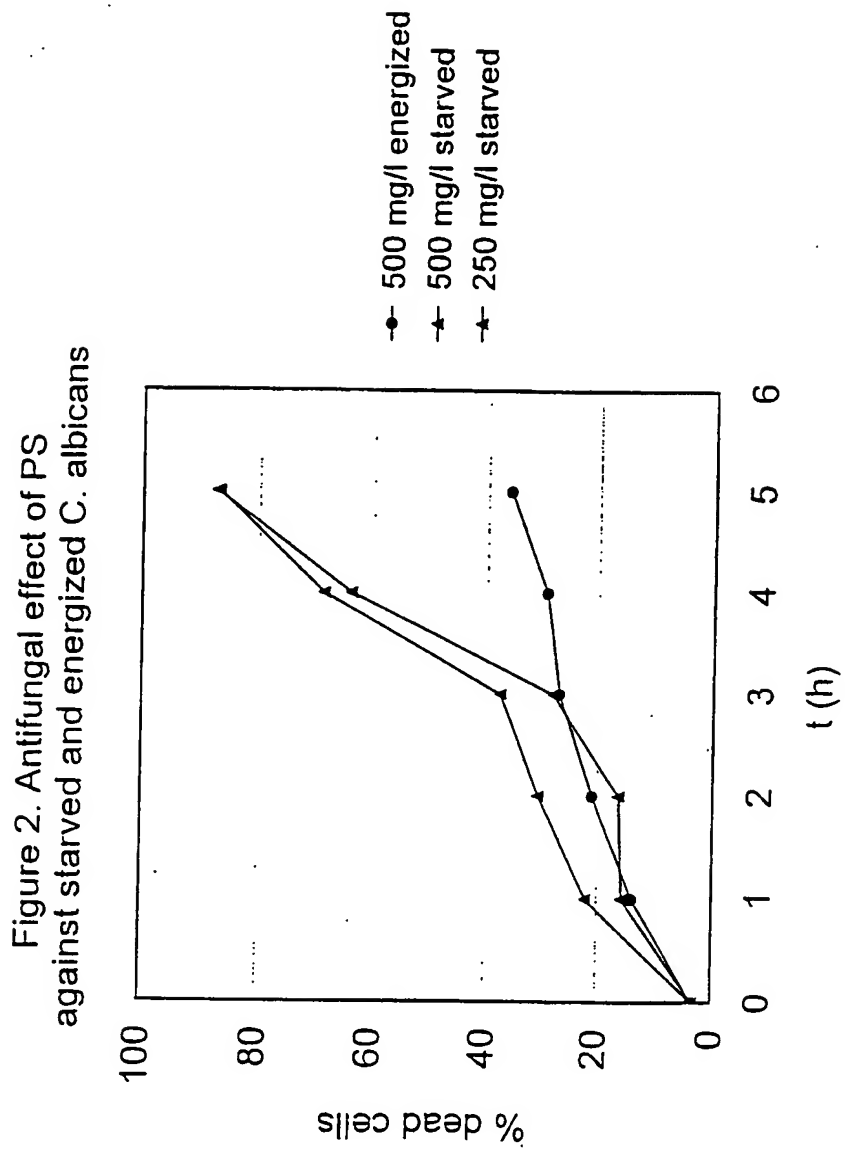


Figure 2. Antifungal effect of PS against starved and energized C. albicans

Figure 3a. Comparison of PS and its salts against C. albicans

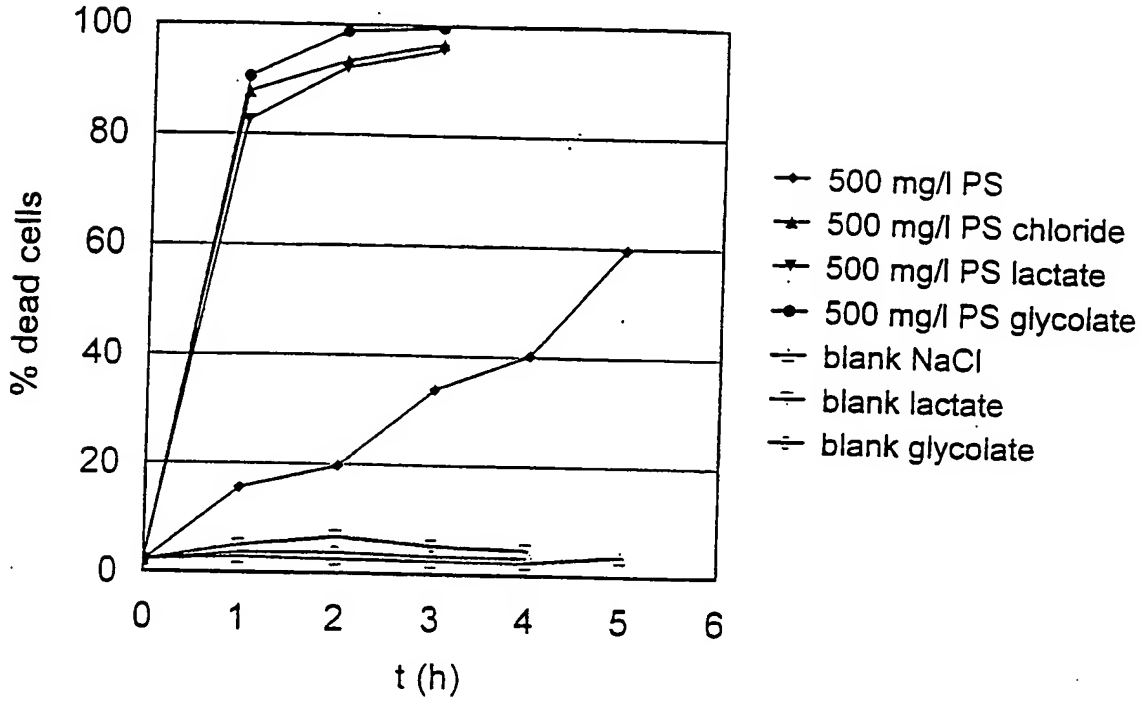


Figure 3b. Comparison of potency of chloride and free base against C. albicans

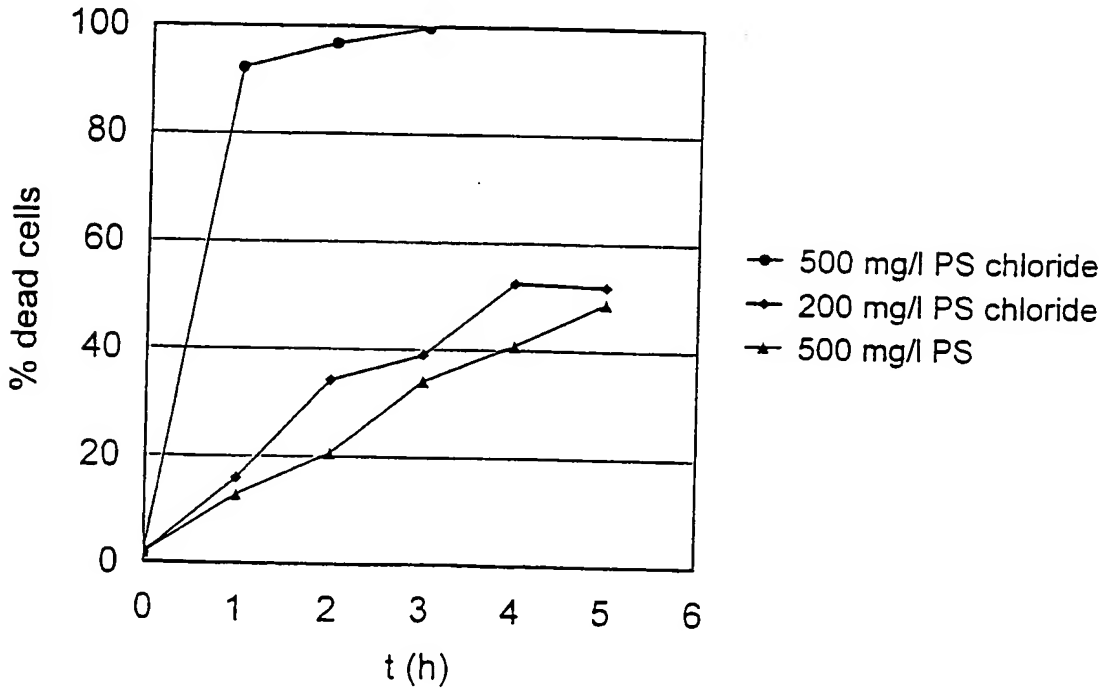


Figure 4a. Antifungal activity in water against C. albicans

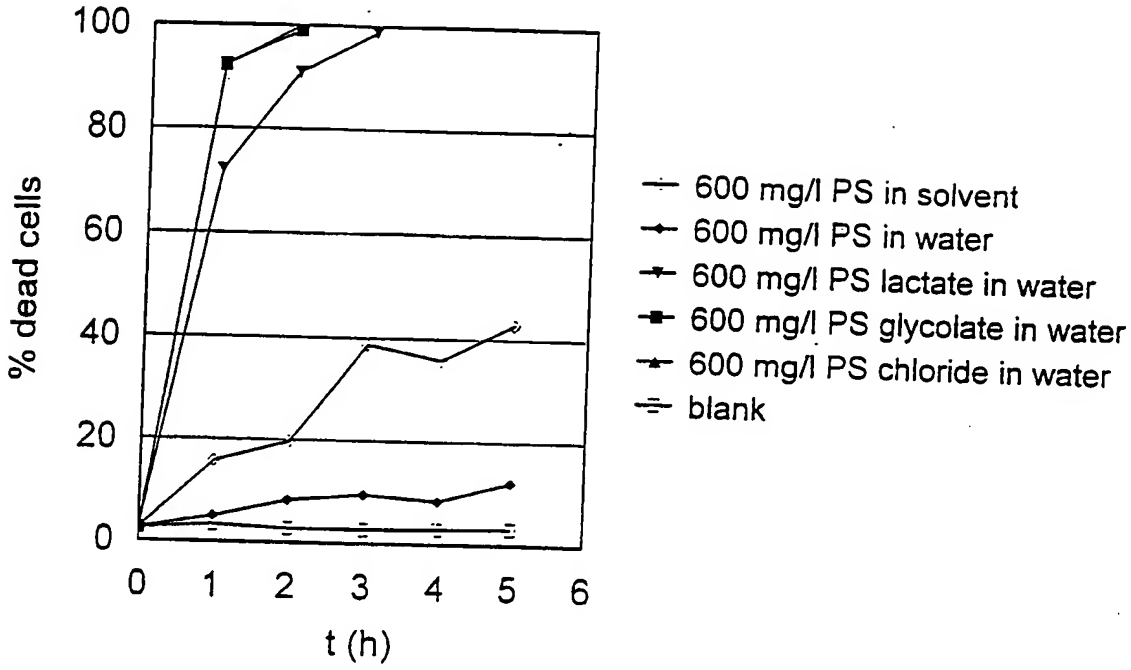
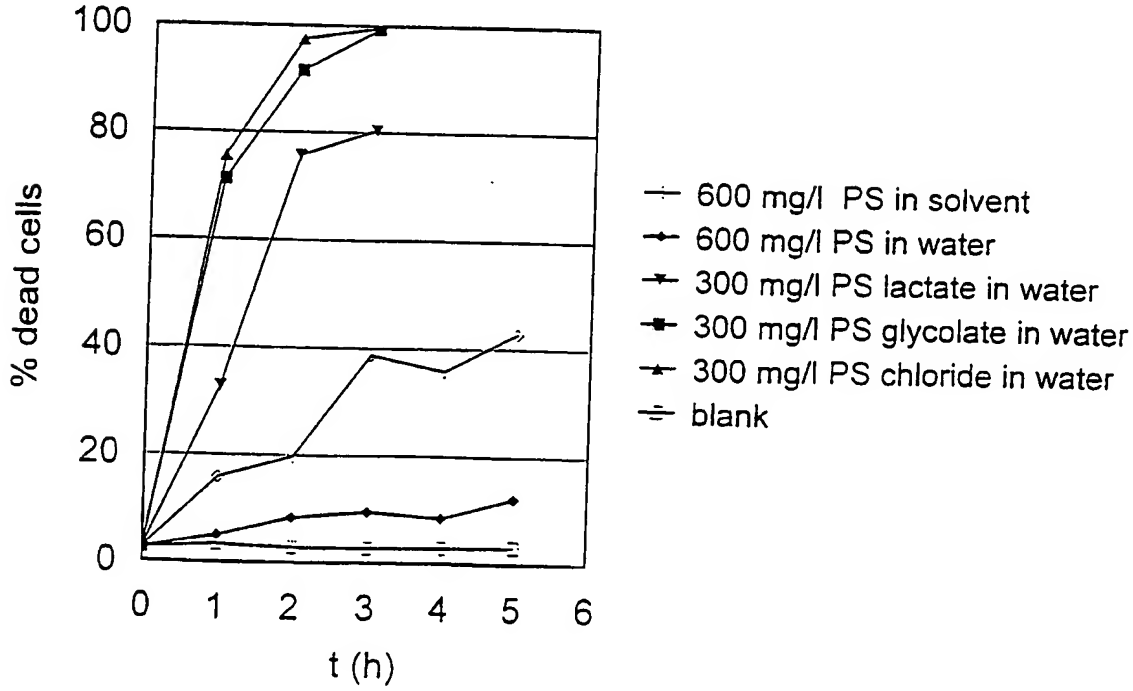


Figure 4b. High potency of PS salts in water against C. albicans



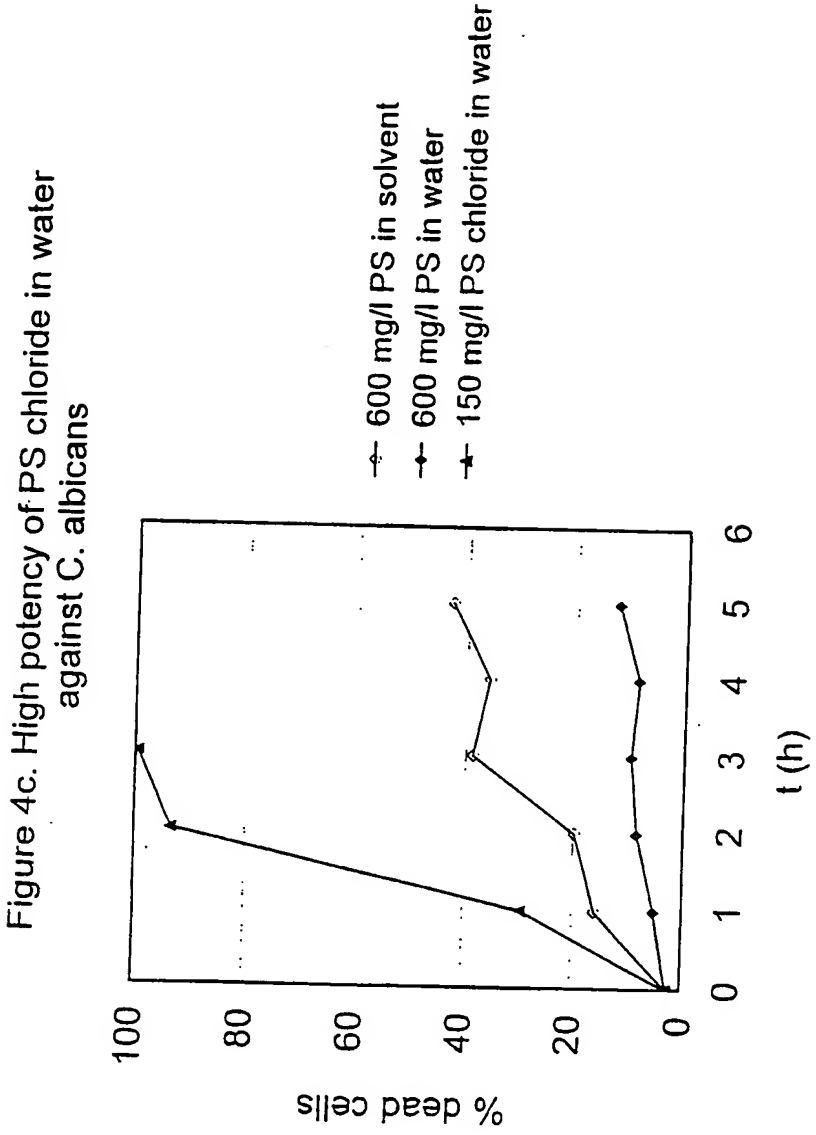


Figure 4c. High potency of PS chloride in water against C. albicans

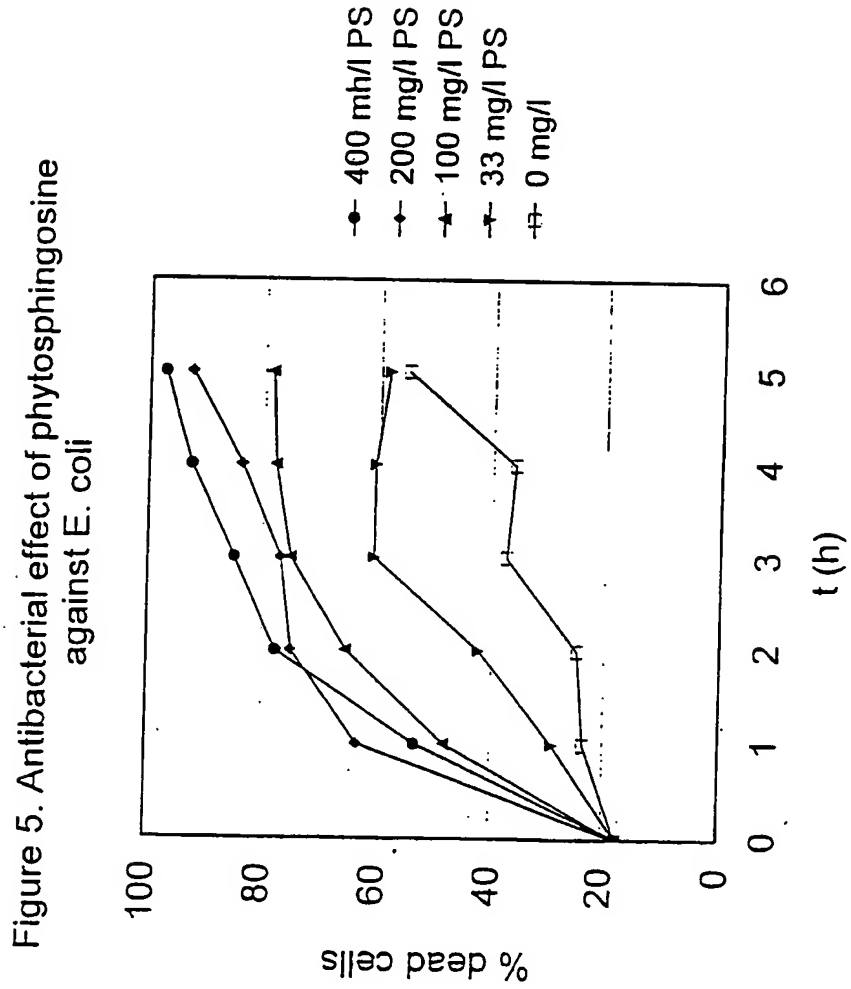
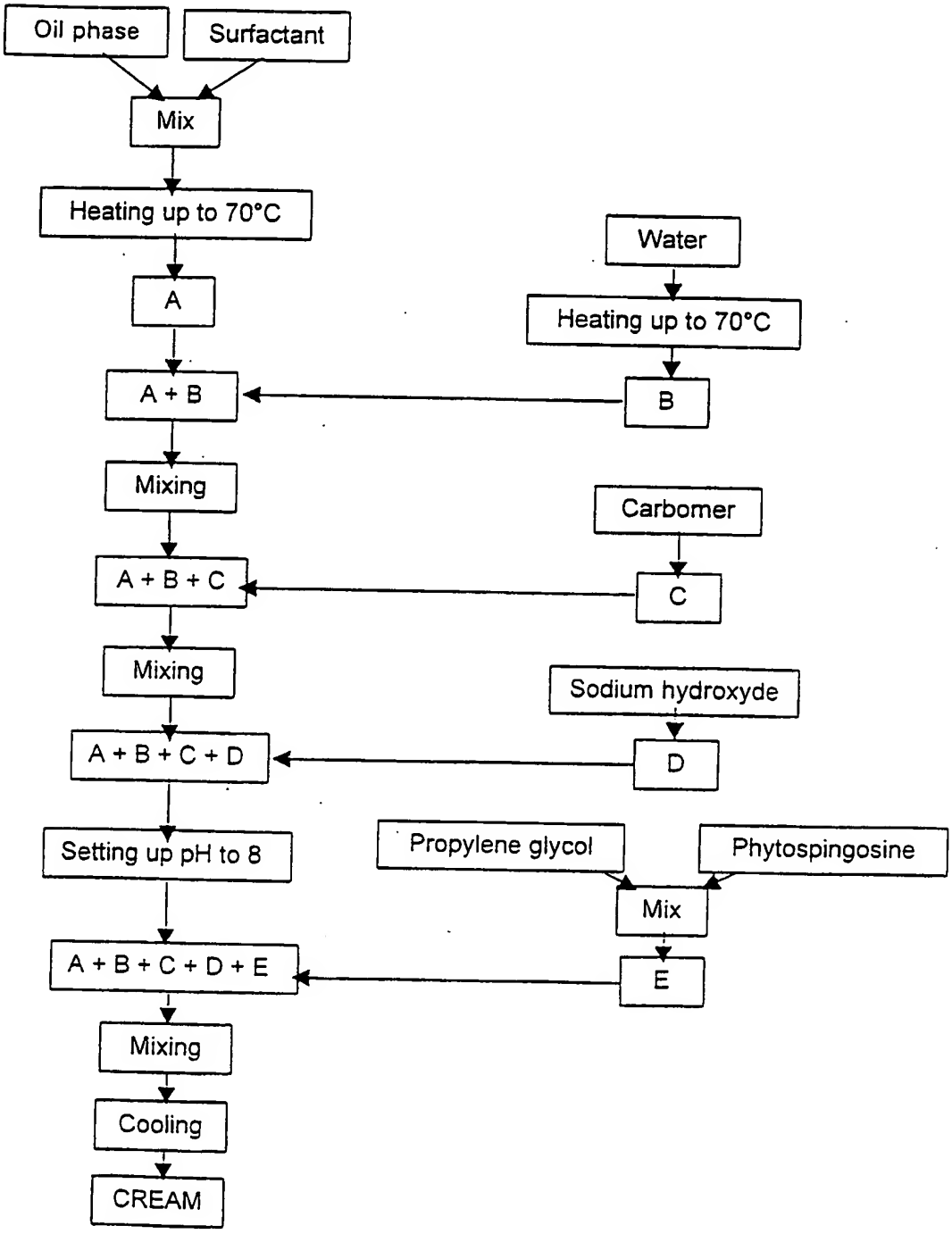


Figure 5. Antibacterial effect of phytosphingosine against E. coli

Figure 6. Flowsheet for preparation of Phytosphingosine containing-formulation



INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/EP 00/02191

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07C215/24 A61K31/133 A61K7/48 A61K7/06 A61P31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07C A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, WPI Data, PAJ, BEILSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the International search report
16 June 2000	06/07/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Pauwels, G

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